Intrathecal transplantation of bone marrow stromal cells attenuates blood-spinal cord barrier disruption induced by spinal cord ischemia-reperfusion injury in rabbits

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Objective: **Intrathecal administration of bone marrow stromal cells has been found to produce beneficial effects on ischemia-reperfusion injury to the spinal cord. The blood-spinal cord barrier is critical to maintain spinal cord homeostasis and neurologic function. However, the effects of bone marrow stromal cells on the blood-spinal cord barrier after spinal cord ischemia-reperfusion injury are not well understood. This study investigated the effects and possible mechanisms of bone marrow stromal cells on blood-spinal cord barrier disruption induced by spinal cord ischemia-reperfusion injury.**
Methods: **This was a prospective animal study conducted at the Central Laboratory of the First Affiliated Hospital, China Medical University. The study used 81 Japanese white rabbits (weight, 1.8-2.6 kg). Spinal cord ischemia-reperfusion injury was induced in rabbits by infrarenal aortic occlusion for 30 minutes. Two days before the injury was induced, bone marrow stromal cells (1×10^8 in 0.2-mL phosphate-buffered saline) were transplanted by intrathecal injection. Hind-limb motor function was assessed using Tarlov criteria, and motor neurons in the ventral gray matter were counted by histologic examination. The permeability of the blood-spinal cord barrier was examined using Evans blue (EB) and lanthanum nitrate as vascular tracers. The expression and localization of tight junction protein occludin were assessed by Western blot, real-time polymerase chain reaction, and immunofluorescence analysis. Matrix metalloproteinase-9 (MMP-9) and tumor necrosis factor-α (TNF-α) expression were also measured.**
Results: **Intrathecal transplantation of bone marrow stromal cells minimized the neuromotor dysfunction and histopathologic deficits ($P < .01$) and attenuated EB extravasation at 4 hours (5.41 ± 0.40 vs 7.94 ± 0.36 μg/g; $P < .01$) and 24 hours (9.03 ± 0.44 vs 15.77 ± 0.89 μg/g; $P < .01$) after spinal cord ischemia-reperfusion injury. In addition, bone marrow stromal cells treatment suppressed spinal cord ischemia-reperfusion injury-induced decreases in occludin ($P < .01$). Finally, bone marrow stromal cells reduced the excessive expression of MMP-9 and TNF-α ($P < .01$).**
Conclusions: **Pre-emptive intrathecal transplantation of bone marrow stromal cells stabilized the blood-spinal cord barrier integrity after spinal cord ischemia-reperfusion injury in a rabbit model of transient aortic occlusion. This beneficial effect was partly mediated by inhibition of MMP-9 and TNF-α and represents a potential therapeutic approach to mitigating spinal cord injury after aortic occlusion. (J Vasc Surg 2013;58:1043-52.)**

Clinical Relevance: **Clinical thoracoabdominal aorta surgery may trigger spinal cord ischemia-reperfusion injury, resulting in paraplegia as well as bladder, bowel, and sexual dysfunction. Transplantation of bone marrow stromal cells has attracted increasing attention in the field of nervous system protection, but its mechanisms have not been elucidated completely. The blood-spinal cord barrier plays a crucial role to maintain normal spinal cord function. This study suggested that intrathecal transplantation of bone marrow stromal cells stabilized blood-spinal cord barrier integrity through inhibiting the upregulation of matrix metalloproteinase-9 and tumor necrosis factor-α and ameliorated spinal cord ischemia-reperfusion injury. This may provide a novel train of thought to enhance the protective effects of bone marrow stromal cells on spinal cord injury.**

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Spinal cord ischemia is a well-recognized complication of acute aortic occlusion for resuscitation in the setting of shock or for the purposes of elective or emergency aortic reconstruction.[1,2] Bone marrow stromal cells (also called *mesenchymal stem cells*) comprise a heterogeneous population of cells located in bone marrow. Bone marrow stromal cells have been transplanted into the intrathecal space in an attempt to maintain spinal cord function in the setting of an acute contusion and hemisection injury, as well as injury resulting from ischemia and reperfusion.[3-6] Although not well-characterized, the possible beneficial effect of bone marrow stromal cells may be related to differentiation,[3] secretion of neurotrophic and antiapoptotic factors,[7,8] and promotion of local neonatal angiogenic factors.[6]

In the setting of trauma, alteration in the blood-spinal cord barrier allows a variety of harmful molecules and proteins to enter the cord parenchyma from the blood stream.[9] Several neurochemicals have been implicated in disruption of the blood-spinal cord barrier after cord injury, among them matrix metalloproteinase-9 (MMP-9) and tumor necrosis factor-α (TNF-α).[10,11] These substances alter cord microvascular permeability and result in cellular ischemia, inflammation, and edema that worsen the initial cord injury. In addition, breakdown of the blood-spinal cord barrier appears to play an important role in regeneration and repair of the spinal cord after trauma.[12]

The role of intrastriatal transplantation of bone marrow stromal cells has been examined in the setting of cerebral ischemia in rats.[13] That study showed that bone marrow stromal cells restored cerebral blood flow, stabilized the blood-brain barrier, and ultimately, improved the outcome after stroke. However, little is known about the effects of bone marrow stromal cells on the blood-spinal cord barrier disruption after spinal cord ischemia. Occludin, a tight junction (TJ) protein that maintains blood-brain barrier and blood-spinal cord barrier integrity, has been reported to be a sensitive indicator of the functional state of the blood-brain barrier.[14]

The primary objective of this study was to investigate potential protective effects of bone marrow stromal cells on the blood-spinal cord barrier and occludin levels in a rabbit model of ischemia reperfusion. Secondary objectives were to evaluate the role of MMP-9 and TNF-α in ischemia-induced blood-spinal cord barrier breakdown with and without pretreatment with bone marrow stromal cells.

METHODS

All experimental animal procedures were conducted with the approval of the Ethics Committee of China Medical University in accordance with *Guide for the Care and Use of Laboratory Animals* (U.S. National Institutes of Health publication No. 85-23, National Academy Press, Washington DC, revised 1996).

Animals and surgical procedure. Japanese white rabbits, weighing 1.8 to 2.6 kg, were used in the study. The rabbits were anesthetized with intravenous 20% ethyl carbamate (1 g/kg) and were allowed to breathe spontaneously. Core body temperature was continuously monitored with a rectal probe and was maintained at 38.5° ± 0.5°C with the aid of a heat lamp. Two catheters were inserted into the ear and femoral artery to measure proximal and distal blood pressure. Lidocaine (0.5%) was administered at the site of the skin incision as a local anesthetic. The infrarenal abdominal aorta was exposed through a transperitoneal approach. After systemic heparinization (200 U/kg), spinal cord ischemia was induced by cross-clamping the abdominal aorta just 1 cm distal to the left renal artery. The clamp was released after 30 minutes.

Bone marrow stromal cells culture and intrathecal injection. Bone marrow was harvested aseptically from tibias of 2-month-old rabbits, and the nucleated cells were isolated by density gradient centrifugation using Percoll (1.073 g/mL; Sigma, St. Louis, Mo). The cells were plated in growth medium consisting of Dulbecco modified Eagle's medium/F12 supplemented with 20% fetal bovine serum and benzylpenicillin (1×10^5 U/mL). Bone marrow stromal cells were easily isolated in the medium because of their tendency to adhere to plastic. After 3 days, the flasks were washed twice with phosphate-buffered saline (PBS) to remove nonadherent cells. The remaining cells were fed every third day.

Cultures of bone marrow stromal cells were maintained at 50% confluence and were passaged three times. According to the detection of flow cytometric plots, third-generation cells are considered to be bone marrow stromal cells. They expressed on their surface CD44 (hyaluronate receptor), CD29 (β1 integrin), and lacked the expression of CD45 and CD34, the phenotype of hemopoietic cells.[15] The bone marrow stromal cells were harvested by trypsinization and were resuspended in PBS before intrathecal injection.

After anesthesia with ethyl carbamate, the intervertebral space between L5 and L6 was punctured with a 16-gauge needle, and polyethylene-10 tubing was inserted through it into the subarachnoid space. The desired position of the catheter was confirmed by cautious aspiration of cerebrospinal fluid. After intrathecal injection of 1×10^8 bone marrow stromal cells in 0.2 mL total fluid volume PBS, or the same volume of PBS (for sham group), the catheter was removed. The animals were placed head-up for 60 minutes. The animals were included in the study only if they had a normal hind-limb motor function just before spinal cord ischemia-reperfusion injury was induced.

Experimental protocol. Rabbits were assigned to four groups randomly by means of a random number table. Group sham (n = 15) was given an intrathecal injection of PBS, and the abdominal aorta was exposed, but without occlusion. Group sham + bone marrow stromal cells (BMSCs; n = 12) received an intrathecal injection of bone marrow stromal cells and the abdominal aorta was exposed, without occlusion. Group ischemia-reperfusion (IR; n = 27) received intrathecal PBS and subsequently underwent to 30 minutes of spinal cord ischemia. Group IR + BMSCs (n = 27) received an intrathecal injection of bone marrow stromal cells and underwent 30 minutes of

spinal cord ischemia. In all groups, the aortic exposure or cross-clamping for 30 minutes was performed 2 days after the experimental intrathecal injection.

Neurologic assessment. At 4 and 24 hours after injury, hind limb motor function was assessed by two blinded observers using a modified Tarlov scale[16-18]: 0, paraplegic with no evident lower extremity motor function; 1, poor lower extremity motor function, weak antigravity movement only; 2, moderate lower extremity function with good antigravity strength but inability to draw legs under body; 3, excellent motor function with the ability to draw legs under body and hop, but not normally; and 4, normal motor function.

Histologic study. All animals were euthanized by a lethal injection of pentobarbital (200 mg/kg) 24 hours after ischemia-reperfusion injury, and the spinal cords (L4-L6) were quickly removed. Paraffin-embedded sections (4 μm) were stained with hematoxylin and eosin. In cases in which the cytoplasm was diffusely eosinophilic, the large motor neuron cells were considered to be necrotic or dead. When the cells demonstrated basophilic stippling (containing Nissl substance), the motor neuron cells were considered to be viable or alive. The intact motor neurons in the ventral gray matter were counted by a blinded investigator in three sections for each rabbit, and the results were averaged.

Measurement of Evans blue extravasation. Blood-spinal cord barrier permeability was determined by measuring Evans blue (EB) extravasation. After ischemia-reperfusion injury, 2% EB dye (10 mL/kg; Sigma) was slowly intravenously administered after ischemia-reperfusion injury. After the EB circulated for 1 hour, the rabbits were anesthetized and perfused through the ascending aorta with 500 mL/kg saline, and the spinal cords (L4-L6) were removed. EB content and EB fluorescence were the two methods used to measure EB extravasation:

First, the spinal cord tissue was weighed and soaked in methanamide for 24 hours (60°C) and then centrifuged. The absorption of the supernatant was measured at 632 nm with a microplate reader (BioTek, Winooski, Vt). The content of EB was measured as micrograms per gram of spinal cord tissue by standardized curve.

Second, the spinal cord tissue was fixed in 4% paraformaldehyde and sectioned (10 μm) and kept frozen and sealed in a light-tight container before measurement of dye fluorescence. EB staining was visualized using a BX-60 (Olympus, Melville, NY) fluorescence microscope (green zone).

Transmission electron microscope study of blood-spinal cord barrier permeability. At 24 hours after ischemia-reperfusion injury, the rabbits were anesthetized and perfused with 500 mL/kg saline rapidly through the ascending aorta, followed with 500 mL/kg fixative (4% glutaraldehyde-2% lanthanum nitrate-0.1M sodium cacodylate trihydrate) over 20 minutes. The spinal cords (L4-L6) were removed and cut into 1-mm^3 pieces. The isolated tissues were immersed in the above fixative for 2 hours and then washed three times in 2% lanthanum nitrate-0.1M sodium cacodylate trihydrate. The tissues were immersed in 1% osmium tetroxide for 2 hours and then washed with sodium cacodylate trihydrate. The sections were dyed with acetic acid, uranium, and lead, mounted on copper grids (200 meshes), and examined under a JEM-1200 EX transmission electron microscope (Joel, Tokyo, Japan). The integrity of the blood-spinal cord barrier was assessed by lanthanum nitrate extravasation.

Immunofluorescence staining of the TJ protein. To identify the TJ protein occludin colocalized with vascular endothelial cells, double immunofluorescence labeling for occludin with CD31 was performed. Each section was first treated with the primary mouse anti-CD31 (dilution 1:20; Abcam, Cambridge, Mass) with donkey anti-mouse Alexa Fluor 594 (dilution 1:500; Invitrogen, Carlsbad, Calif), and was followed by mouse anti-occludin-fluorescein isothiocyanate conjugate (dilution 1:50; Invitrogen, Camarillo, Calif) and analyzed by a Leica TCS SP2 (Leica Microsystems, Bannockburn, Ill) laser scanning spectral confocal microscope.

Western blot. The expression of occludin, MMP-9, and TNF-α in spinal cord tissue was determined by Western blot analyses performed as previously described.[19] The antibodies used in this experiment were mouse monoclonal antioccludin (Invitrogen), mouse monoclonal anti-MMP-9 (Abcam), rabbit polyclonal anti-TNF-α (Bioss, Beijing, China), and horseradish peroxidase-conjugated secondary antibodies (Bioss). Semiquantitation of scanned images was performed using Quantity One software (Bio-Rad Laboratories, Milan, Italy).

Real-time PCR. Total RNA was extracted using a TRIzol Kit and converted to first-strand complementary DNA according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green SuperMix-UDG in a Prism 7000 Q real-time PCR detection system (Applied Biosystems, Foster City, Calif). The primer sequences used for PCR were:

Occludin (218 bp): forward 5′-GCT TCT GGA TCT ATG TAT GGC TCA C-3′; reverse 5′-TCA TAG CGG TCC ATC TTT CTT CGA G-3′

MMP-9 (119 bp): forward 5′-TGT GTC TTC CCC TTC GTC TT-3′; reverse 5′-CCC CAC TTC TTG TCG CTG T-3′

Glyceraldehyde-3-phosphate dehydrogenase (177 bp): forward 5′-TCG GCA TTG TGG AGG GGC TC-3′; reverse 5′-TCC CGT TCA GCT CGG GGA G-3′

Amplification was performed by using the following cycles: 50°C for 2 minutes (uracil-DNA glycosylase incubation), 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing at 60°C for 30 seconds. All reactions were performed in triplicate. Melting curve analysis was performed to ensure the specificity of quantitative PCR. Data analysis was performed using the 2-ΔΔCT method described by Livak,[20] where glyceraldehyde-3-phosphate dehydrogenase was used as reference gene.

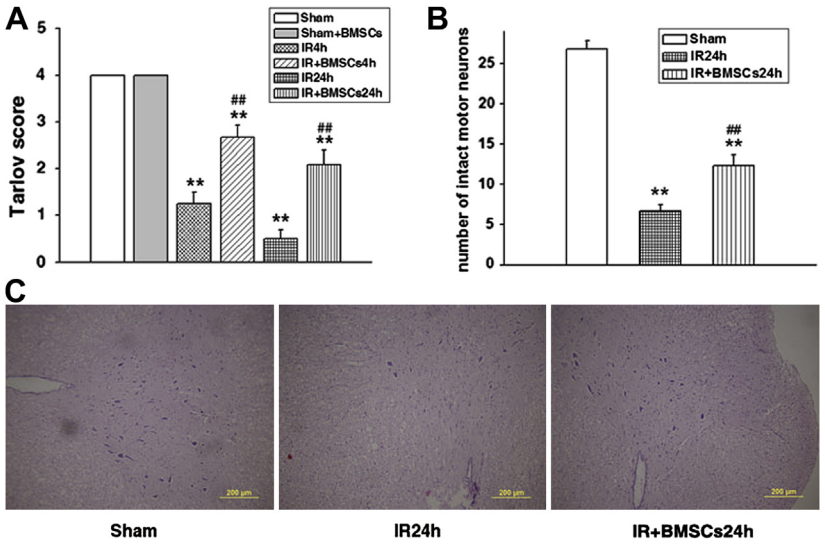

Fig 1. Effects of bone marrow stromal cells (*BMSCs*) on neurologic motor function and histologic assessment of the spinal cord after spinal cord ischemia-reperfusion (*IR*) injury. **A,** Tarlov score at 4 and 24 hours after injury (n = 12). **B,** Number of intact motor neurons in the ventral gray matter. $^{**}P < .01$ vs group sham; $^{\#\#}P < .01$ vs group IR. Data are presented as mean ± standard error (n = 6). **C,** Representative sections of lumbar spinal cords stained with hematoxylin and eosin (original magnification, ×100).

Statistical analysis. Values are expressed as mean ± standard error. Statistical analyses were performed with one-way analysis of variance with post hoc Bonferroni test. $P < .05$ was considered to be statistically significant.

RESULTS

Neurologic assessment. All rabbits showed normal hind limb motor function 2 days after intrathecal injection before the induction of ischemia. The individual neurologic scores of the four groups at 4 and 24 hours after reperfusion are shown in Fig 1, *A*. The sham and sham + BMSCs animals retained a normal motor function of lower limbs throughout the observation period. A 30-minute period of infrarenal aortic occlusion resulted in severe lower extremity neurologic deficits in the control rabbits, and prophylactic transplantation of bone marrow stromal cells enhanced the recovery of motor function (group IR + BMSCs vs group IR; $P < .01$, at 4 and 24 hours respectively).

Histologic assessment. A 30-minute ischemia resulted in significant loss of the motor neurons in the control animals at 24 hours after the operation ($P < .01$ vs group sham; Fig 1, *B*). In contrast, more intact motor neurons were found in the lumbar spinal cords of group IR + BMSCs ($P < .01$ vs group IR; Fig 1, *B*). In group sham and group sham + BMSCs, the lumbar spinal cord was intact, and more large motor neurons were present in the anterior horn than in group IR (Fig 1, *C*).

Treatment with bone marrow stromal cells decreases blood-spinal cord barrier leakage. The permeability of the blood-spinal cord barrier was measured by EB extravasation, which was visualized as red fluorescence under the fluorescent microscope. In sham rabbits, bone marrow stromal cells had no effect on the blood-spinal cord barrier. However, pre-emptive treatment with bone marrow stromal cells significantly decreased blood-spinal cord barrier leakage after spinal cord ischemia-reperfusion injury (Fig 2, *A-F*). Meanwhile, EB content was less at 4 and 24 hours in group IR + BMSCs (5.41 ± 0.40 and 9.03 ± 0.44 μg/g) than in group IR (7.94 ± 0.36 and 15.77 ± 0.89 μg/g; $P < .01$; Fig 2, *G*).

As an electron-opaque tracer, lanthanum ion is widely used to examine the integrity of the blood-brain barrier by transmission electron microscope.[19] As shown in Fig 3, in sham spinal cord specimens, the lanthanum nitrate was exclusively localized in the capillary. There was no permeation of the lanthanum nitrate into the basement membrane and beyond the microvessels. At 24 hours after ischemia-reperfusion injury, a leakage of lanthanum nitrate was found in the basement membrane, even invading into the parenchyma along the TJ in group IR, whereas the extravasation of lanthanum was reduced in group IR + BMSCs.

Treatment with bone marrow stromal cells suppressed a decrease in occludin. Western blot analysis indicated that the decrease in occludin after spinal cord ischemia-reperfusion injury was inhibited by transplanted bone marrow stromal cells ($P < .01$; Fig 4, *A*). Similarly, real-time PCR showed bone marrow stromal cells increased messenger RNA expression of occludin, which was reduced by spinal cord ischemia-reperfusion injury ($P < .01$; Fig 4, *B*). Double immunofluorescence labeling for occludin with vascular endothelial cells revealed that spinal

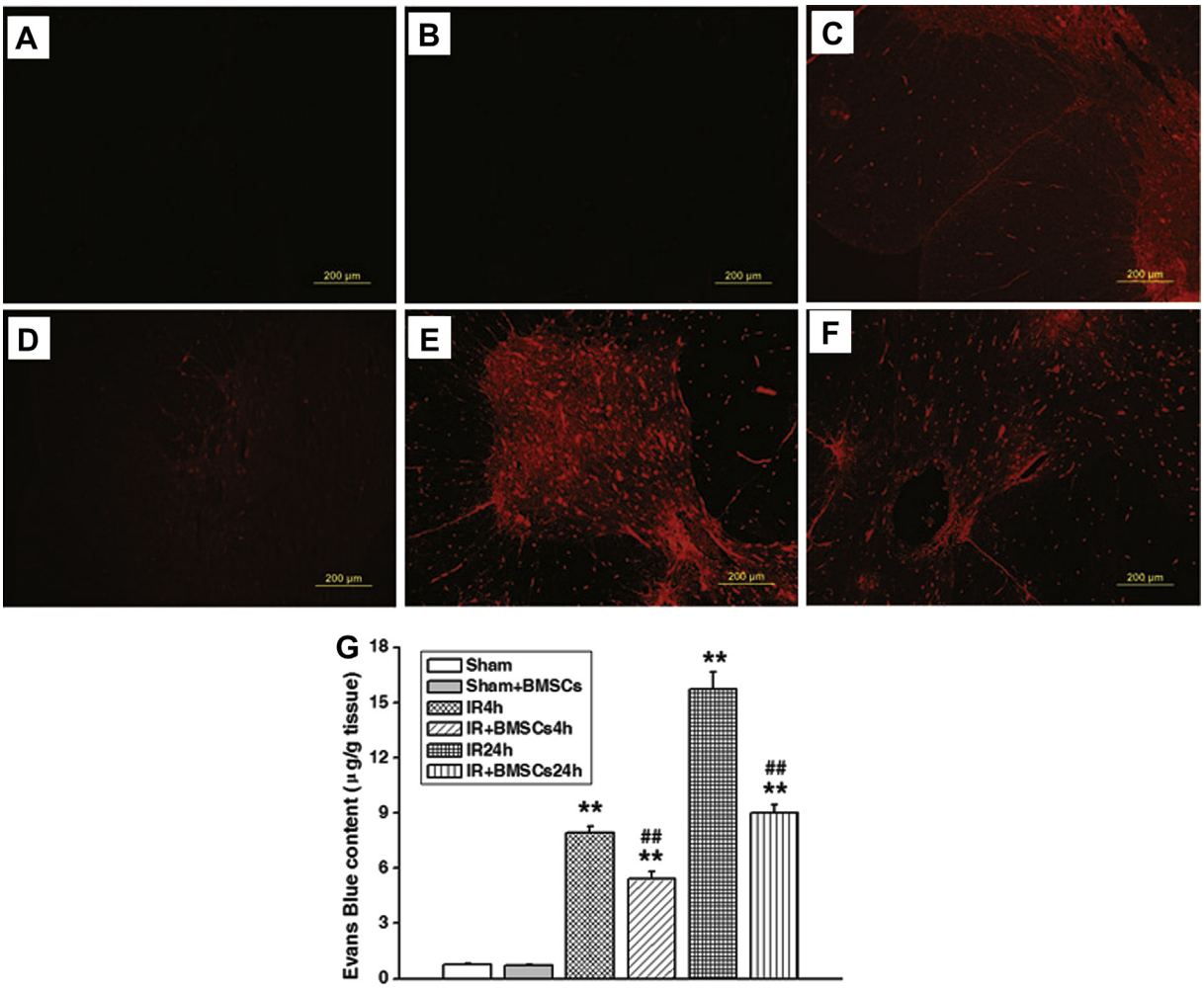

Fig 2. Effects of bone marrow stromal cells (*BMSCs*) on Evans blue (EB) extravasation after spinal cord ischemia-reperfusion (*IR*) injury. Almost no red fluorescence was seen in spinal cord parenchyma in **(A)** group sham and **(B)** group sham + BMSCs. **C,** Much red fluorescence could be seen at 4 hours after injury. **E,** This turned stronger at 24 hours after injury, especially in gray matter. **D** and **F,** In contrast, EB red fluorescence was significantly weakened in group IR + BMSCs. **G,** Quantification data of EB content of spinal cord (μg/g) are presented as mean ± standard error (n = 6). $^{**}P < .01$ vs group sham; $^{\#\#}P < .01$ vs group IR.

cord ischemia-reperfusion injury led to a decrease and discontinuous arrangement of occludin-positive protein, which can be ameliorated by pretreatment with bone marrow stromal cells (Fig 4, *C*).

Treatment with bone marrow stromal cells inhibited upregulation of MMP-9. Western blot analysis indicated that the level of MMP-9 increased after injury, and transplantation of bone marrow stromal cells inhibited the upregulation of MMP-9 ($P < .01$; Fig 5, *A*). Similarly, real-time PCR showed bone marrow stromal cells decreased messenger RNA expression of MMP-9, which was increased by spinal cord ischemia-reperfusion injury ($P < .01$; Fig 5, *B*).

Treatment with bone marrow stromal cells reduced TNF-α expression. Western blot revealed that TNF-α was expressed at very low levels in the sham group, was highly expressed after spinal cord ischemia-reperfusion injury, and was reduced by transplantation of bone marrow stromal cells ($P < .01$; Fig 5, *C*).

DISCUSSION

The current investigation is among the first to demonstrate the effectiveness of bone marrow stromal cells in reducing inflammation of the blood-spinal cord barrier after ischemia-reperfusion injury. Findings from this study show that bone marrow stromal cells administered in the intrathecal space before ischemic injury reduce blood-spinal cord barrier permeability as measured by EB and lanthanum nitrate extravasation. Results from this work also demonstrate that when administered in this pre-emptive manner, bone marrow stromal cells preserve the

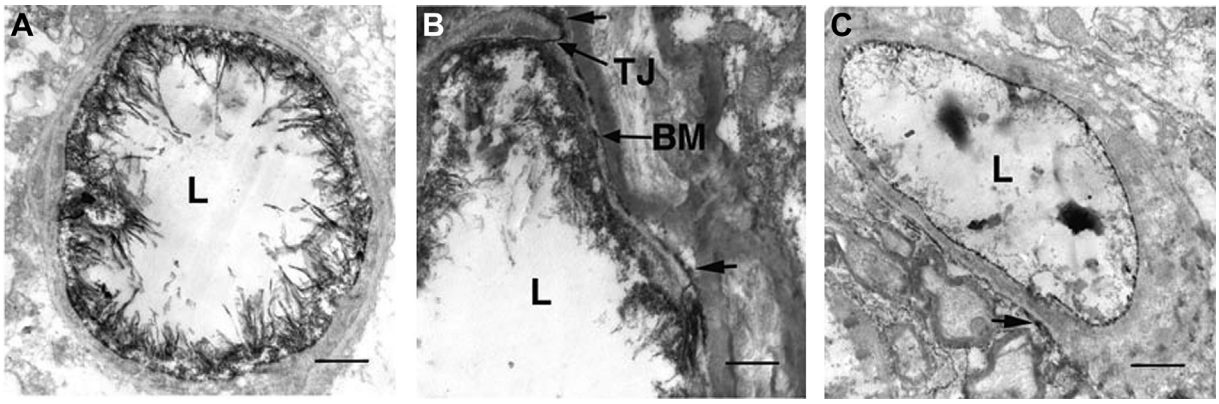

Fig 3. Effects of bone marrow stromal cells (BMSCs) on the ultrastructure of the blood-spinal cord barrier by transmission electron microscopy at 24 hours after spinal cord ischemia-reperfusion (IR) injury. **A,** In group sham, the lanthanum nitrate was exclusively localized in the capillary. **B,** In group IR, a leakage of lanthanum was found in the basement membrane (*BM*), even invading into the parenchyma along the tight junction (*TJ*). **C,** In group IR + BMSCs, only little extravasation of lanthanum was observed. The *unlabeled arrowheads* indicate the leakage of lanthanum. *L*, Capillary lumen. *Scale bar*: 1 μm.

number of intact motor neurons and extremity motor function after ischemia-reperfusion injury to the spinal cord.

Compared with embryonic stem cells and neural stem cells, bone marrow stromal cells can be readily harvested and grown in cell culture. Moreover, autologous transplantation of bone marrow stromal cells would circumvent potential ethical and immune rejection considerations. Bone marrow stromal cells may be candidates for treating acute spinal cord ischemic injuries. In addition to their multilineage differentiation potential and antiapoptotic and immunomodulatory effects, bone marrow stromal cells can also suppress inflammation.[21] Inflammatory factors play a critical role in disrupting the blood-spinal cord barrier.

Three bone marrow stromal cell transplantation protocols have been used in spinal cord injury. Previously, most transplantations were carried out by direct local delivery of cells into the spinal cord.[3] However, this highly invasive technique may cause additional damage and is difficult to translate to human patients. Another transplantation protocol used the intravenous route, which is safe and convenient, but a large number of cells are required. Intrathecal transplantation is a novel, minimally invasive method that improves the efficiency of transplantation and enhances the efficacy of the treatment.[22] Thus, intrathecal injection of bone marrow stromal cells appears to be a suitable approach to study their effects of on the blood-spinal cord barrier.

Shi et al[6] examined the therapeutic effect of bone marrow stromal cells on ischemia-injured spinal cord and found that the therapeutic time window was crucial to achieving therapeutic efficiency (earlier better than late). They also confirmed that prophylactic intrathecal transplantation of bone marrow stromal cells played a potential role in preventing neurologic injury after spinal cord ischemia.[5] Early neural damage and degeneration was related to blood-brain barrier breakdown. Therefore, early restoration of the barrier could alleviate neuropathologic damage and facilitate recovery. Even better, prophylactic treatment to maintain a healthy blood-brain barrier has the potential to delay the neurodegeneration occurance.[23] Jacobs et al[24] reported severe vascular permeability disruption appeared as early as 30 minutes after spinal cord ischemia-reperfusion in rabbits. To play the role of bone marrow stromal cells against blood-spinal cord barrier disruption as soon as possible, bone marrow stromal cells were intrathecally transplanted 2 days before spinal cord ischemia-reperfusion injury in the current study.

The current findings demonstrated that EB extravasation was increased at 24 hours after reperfusion; meanwhile, neurologic function and surviving motor neurons decreased, compared with 4 hours, consistent with the previous study.[24] It demonstrates neurologic deterioration after spinal cord ischemia-reperfusion injury is associated with breakdown of blood-spinal cord barrier integrity. Sharma et al[25] suggest the capillaries are more numerous in the spinal gray matter, where they form a dense capillary bed, compared with the white matter. These findings are consistent with the current observation that EB extravasation was greater in the gray matter.

The present study focused on the protective role of the TJ protein occludin in attenuating the blood-spinal cord barrier disruption. Occludin was the first TJ transmembrane molecule discovered, and degradation of occludin contributes to blood-brain barrier disruption in the early stages of ischemic stroke.[26] Moreover, early blood-brain barrier permeability detected by quantitative magnetic resonance imaging after ischemic stroke is related to occludin phosphorylation.

In the current study, the permeability of the blood-spinal cord barrier increased after spinal cord ischemia-reperfusion injury concurrent with a decrease in occludin

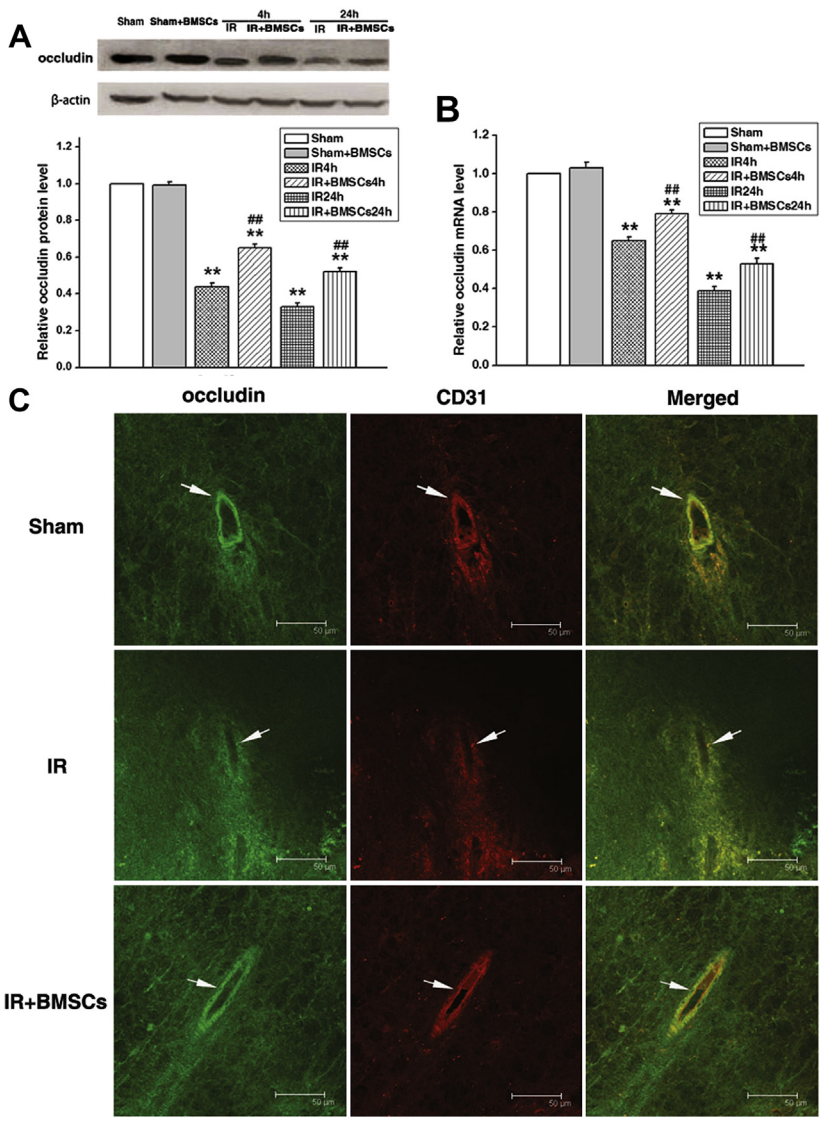

Fig 4. Bone marrow stromal cell (*BMSC*) transplantation suppressed the decreases in the tight junction protein occludin after spinal cord ischemia-reperfusion (*IR*) injury. **A,** Representative Western blot and quantitative protein analysis of occludin in the spinal cord at 4 and 24 hours after injury. **B,** Real-time polymerase chain reaction analysis shows occludin messenger RNA (*mRNA*) expression. The expression level of each sample is expressed as the ratio to group sham. $^{**}P < .01$ vs group sham; $^{\#\#}P < .01$ vs group IR. Data are presented as mean ± standard error (n = 6). **C,** Immunofluorescence photomicrographs of occludin (*green*) along microvasculature (*red*) 24 hours after injury.

messenger RNA and protein level. The immunofluorescence intensity of occludin along the microvasculature also appeared to be reduced after ischemia-reperfusion injury. Furthermore, electron microscopy showed lanthanum was present in basement membrane and invaded into the parenchyma along the TJ after ischemia-reperfusion injury. By contrast, occludin expression was obviously increased in rabbits treated with bone marrow stromal cells. This effect was also clearly observed by immunofluorescence, in which continuity of occludin staining was markedly increased. Meanwhile, lanthanum extravasation was considerably attenuated.

However, Noble and Wrathall[27] and Sharma et al[28] examined blood-spinal cord barrier leakage at the ultrastructural level using horseradish peroxidase or lanthanum as the tracer in spinal cord contusion and incision injuries, respectively. These investigators reported that an increase in endothelial cell membrane vesicular transport (rather than widening of the TJ) was responsible for blood-spinal cord barrier leakage. The different results may be due to variation in the type of spinal cord insult and differences in the experimental animal model. It is widely acknowledged that blood-brain barrier disruption is related to disruption of TJ in ischemic stroke animal models.[25,29,30]

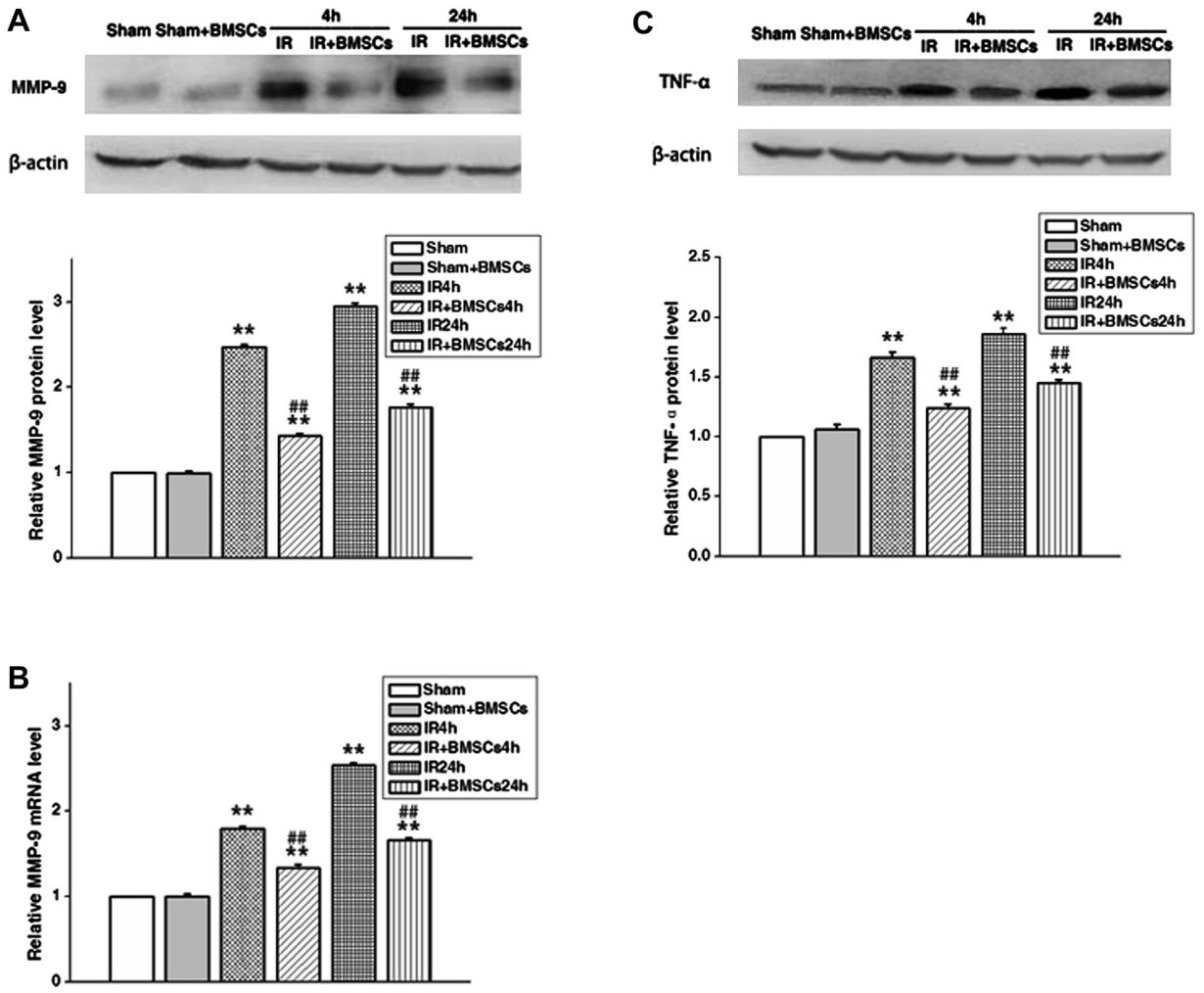

Fig 5. Transplantation of bone marrow stromal cells (*BMSCs*) suppressed the upregulation of matrix metalloproteinase (*MMP*)-9 and tumor necrosis factor (*TNF*)-α after spinal cord ischemia-reperfusion (*IR*) injury. **A,** Representative Western blot and quantitative protein analysis of MMP-9 in spinal cord at 4 and 24 hours after injury. **B,** Real-time polymerase chain reaction analysis of MMP-9 messenger RNA (*mRNA*) expression. **C,** Representative Western blot and quantitative protein analysis of TNF-α in spinal cord at 4 and 24 hours after injury. The expression level of each sample is expressed as the ratio to group sham. $^{**}P < .01$ vs group sham; $^{\#\#}P < .01$ vs group IR. Data are presented as mean ± standard error (n = 6).

Analogous to cerebral ischemia-reperfusion injury, the blood-spinal cord barrier destruction likely has a direct correlation to disruption of TJ after spinal cord ischemia-reperfusion injury, and bone marrow stromal cells protected the blood-spinal cord barrier by reducing the loss of TJ protein.

Excessive MMPs, and in particular MMP-9, significantly destroy barrier integrity by digesting basement membranes and TJ proteins of endothelial cells.[31,32] Another series of experiments showed that MMPs were upregulated and associated with blood-brain barrier or blood-spinal cord barrier dysfunction after stroke[33] and spinal cord contusion.[10] MMP inhibitors have been confirmed to decrease infarct volume[34] and protect the blood-brain barrier[31] in a stroke model.

Several lines of recent evidence support a role of TNF-α in pathologic blood-brain barrier opening in many central nervous system diseases such as focal cerebral ischemia, cerebral trauma, and multiple sclerosis.[35-37] TNF-α induces downregulation of occludin expression to increase blood-brain barrier permeability[38] and is a trigger to induce MMP-9 release from pericytes through mitogen-activated protein kinases and phosphoinositide-3-kinase/Akt.[39]

It has been clarified that bone marrow stromal cells possessed anti-inflammatory properties and significantly decreased TNF-α expression in a lipopolysaccharide-induced inflammation model.[40] This study presented that the permeability of the blood-spinal cord barrier increased, and TJ protein decreased after spinal cord ischemia-reperfusion injury concurrently with MMP-9 and TNF-

α upregulation; meanwhile, pretreatment with bone marrow stromal cells inhibited the elevation of MMP-9, TNF-α, and protected blood-spinal cord barrier and TJ protein, suggesting bone marrow stromal cells may alleviate disruption of the blood-spinal cord barrier via suppression of MMP-9 and TNF-α.

CONCLUSIONS

Pre-emptive transplantation of bone marrow stromal cells stabilized the blood-spinal cord barrier integrity against spinal cord ischemia-reperfusion injury in a rabbit model of transient aortic occlusion. This beneficial effect is partly mediated by inhibition of MMP-9 and TNF-α and represents a potentially novel mode of therapy to reduce ischemia-induced damage to the spinal cord.

AUTHOR CONTRIBUTIONS

Conception and design: FB, WH, MH
Analysis and interpretation: FB, SX, AC, MH
Data collection: FB, WH, LX
Writing the article: FB, MH, PW
Critical revision of the article: FB, LX, TW, PW, MH
Final approval of the article: FB, WH, SX, LX, AC, TW, PW, MH
Statistical analysis: FB, LX
Obtained funding: MH
Overall responsibility: MH

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